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## **FANCD2 protects genome stability by recruiting RNA processing enzymes to resolve R-loops during mild replication stress**

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## Abstract

R-loops, which consist of DNA: RNA hybrids and displaced single-strand DNA, are a major threat to genome stability. We have previously reported that a key Fanconi anemia protein, FANCD2, accumulates on large fragile genes during mild replication stress in a manner depending on R-loops. In this study, we found that FANCD2 suppress R-loop levels. Furthermore, we identified FANCD2 interactions with RNA processing factors, including hnRNP U and DDX47. Our data suggest that FANCD2 that accumulates with R-loops in chromatin recruits these factors, thereby promoting efficient processing of long RNA transcripts. This may lead to reduction of transcription-replication collisions, as detected by PLA between PCNA and RNA Polymerase II, and hence, lowered R-loop levels. We propose that this mechanism might contribute to maintenance of genome stability during mild replication stress.

**Short running title:** FANCD2 and RNA processing factors resolve R-loops

**Key words:** FANCD2, R-loops, hnRNP U, DDX47, replication stress

**A list of defined abbreviations:** FA, Fanconi anemia; MMC, mitomycin C; ICL, interstrand crosslink; DEB, diepoxybutane; PCNA, Proliferating Cell Nuclear Antigen; APH, aphidicolin; UFB, ultra-fine bridges; dsDNA, double-strand DNA; hnRNP U, heterogeneous nuclear ribonucleoprotein U; PLA, proximal ligation assay

## Introduction

Fanconi anemia (FA) is a hereditary disorder characterized by genome instability, and accompanied by various phenotypes including physical abnormalities at birth, small stature, progressive development of hematopoietic stem cell failure, myelodysplasia/leukemia and solid tumors [1]. FA is caused by mutations in any one of 22 FA genes identified to date [2,3]. Cells from FA patients invariably display remarkably elevated levels of mitomycin C (MMC) or DEB-induced chromosomal breakage and complex chromosomal rearrangements such as quadriradial formations. Therefore, the FA gene products are considered to function in a cellular pathway (termed FA pathway) for interstrand crosslink (ICL) repair. FANCD2, together with FANCI (forming the I-D2 complex), is monoubiquitinated by the FA core complex, leading to its accumulation on damaged chromatin (forming microscopically visible “I-D2 foci”). Once localized, the complex coordinates a number of critical activities during ICL repair [4]. For example, FANCD2 is essential for “unhooking” of the ICL and the recruitment of SLX4/XPF nuclease during replication of an ICL-carrying plasmid in xenopus egg extracts [5]. Consistent with this, monoubiquitinated FANCD2 is co-immunoprecipitated with SLX4 in chicken DT40 cells [6]. Second, FANCD2 can orchestrate homologous recombination (HR) by promoting end resection by CtIP nuclease [7,8] or stabilizing nucleoprotein filaments of RAD51 recombinase [9].

Besides these activities, it has been suggested that FANCD2 can function to suppress replication stress and promote restart of a stalled replication fork. FANCD2 was shown to interact with the MCM helicase complex in response to replication stress [10]. In addition to RAD51 and BRCA1/2 proteins, FANCD2 protects stalled replication forks from Mre11-mediated degradation [11,12]. Recent work further indicates that histone H3K4 methylation by the SETD1A-BOD1L complex enforces nucleosome remodeling activity of FANCD2, resulting in suppression of degradation of stalled replication forks via RAD51 filament stabilization [13,14].

Treatment of cells with low-dose aphidicolin (APH), a DNA polymerase inhibitor, triggers replication stress, resulting in breaks and gaps in a set of specific chromosomal locations, termed common fragile sites (CFS; FRA3B, FRA16D, etc) [15,16]. In this situation, very fine bridges between sister chromatids during anaphase (termed ultra-fine bridges, UFB) can be observed by immunostaining with anti-BLM, PICH, or FANCM antibodies, but they are not detected by DNA

staining specific for dsDNA, and are thus presumed to consist primarily of ssDNA. FANCD2 forms sister foci at the roots of UFBs and accumulates at genomic loci containing CFSs during replication stress [17]. Recently, it was reported that FANCD2 is also required for efficient DNA replication across CFSs, and it maintains stability of the CFSs [18].

We previously found, using a ChIP-sequencing approach, that FANCD2 accumulation is mostly restricted to the middle portion of large transcribed genes upon mild replication stress [19]. These large genes often correspond to the known CFSs where transcription complexes may collide with replication forks in S phase, causing formation of R-loops and CFS expression. R-loops, which consist of DNA: RNA hybrids and displaced single-strand DNA, constitute a major threat to genome stability [20]. We also identified that FANCD2 accumulation in chromatin as foci at CFSs is dependent on the formation of R-loops. It is intriguing to note that these huge genes are highly expressed with essential functions in post-mitotic neurons that have ceased DNA replication [21]. Thus, the simultaneous occurrence of transcription and replication across these huge genes is prohibited in neurons. If transcription-replication collisions occur in cycling cells, the FANCD2 function needs to be deployed to reduce the conflicts and replication stress. Indeed, prior studies revealed that FANCD2 is required to suppress R-loop levels that reflect transcription-replication conflicts [18,22,23]. However, how FANCD2 coordinates this function remains unclear.

Our previous attempts to identify FANCD2 interactors revealed that FANCD2 associates with a critical end resection factor, CtIP [7,8]. In the same set of mass spectrometric data, we also identified several RNA processing factors, including heterogeneous nuclear ribonucleoprotein U (hnRNP U)/SAF-A and DDX47(unpublished). It is known that hnRNP U plays multiple important roles, in transcription elongation through an interaction with nuclear actin and RNA polymerase II [24,25], in RNA stability control [26], alternative splicing through regulation of U2snRNP maturation [27], Xist-mediated transcriptional silencing [28], maintenance of chromosome stability [29], and antagonizing R-loop formation after DNA damage [30]. DDX47 encodes a member of the DEAD box protein family characterized by the conserved domain Asp-Glu-Ala-Asp (DEAD) that may function as a putative ATP-dependent RNA helicase, but its precise role remains unknown [31].

In this study, we confirmed the association of these factors with FANCD2 using Co-

immunoprecipitation (Co-IP) as well as a Proximal Ligation Assay (PLA). We also found that R-loop levels are increased in hnRNP U or DDX47 depleted U2OS cells. Given these results, we propose that FANCD2 recruits RNA processing enzymes, including hnRNP U and DDX47, to resolve R-loops, and coordinates transcription-replication collisions so as to prevent replication fork collapse.

## Results

### **FANCD2 attenuates collisions between replication and transcription machinery during mild replication stress**

We have previously reported that FANCD2 accumulates at large transcribed genes, and its accumulation depends on formation of R-loops that may be induced by transcription-replication collisions during mild replication stress [19]. To observe whether transcription-replication collisions occur upon replication stress in our condition, we wished to detect colocalization of RNA polymerase II (Pol II), proliferating cell nuclear antigen (PCNA), and FANCD2 using a proximal ligation assay (PLA). PLA can generate strong signals *in situ*, when two molecules exist within a 40 nm proximity in cells [32]. Thus we reasoned that we would be able to capture the collision events (or persistence of collisions) as well as FANCD2 accumulation in close proximity to the respective transcription and replication machineries using this assay and specific antibodies.

Indeed, higher numbers of PLA signals between Pol II and PCNA were generated in cells treated with APH (Figure 1A), indicating that transcription-replication collision occurs in APH-treated cells. Furthermore, we observed increased numbers of PLA dots after APH stimulation between PCNA and FANCD2 or between FANCD2 and Pol II (Figure 1A), consistent with the possibility that FANCD2 accumulates at the regions where transcription-replication conflicts occur. In these experiments, FANCD2 was detected by anti-FLAG antibody since we used U2OS-D2-FLAG cells that carry a 3x FLAG tag in-frame at the FANCD2 C-terminus. This is also consistent with our previously published observation that PLA between FANCD2 and a DNA: RNA hybrid-specific antibody S9.6 are increased upon replication stress [19]. In *FANCD2*-depleted cells, greater numbers of PLA signals between PCNA and Pol II were observed following APH treatment compared to control cells (Figure 1B). Taken together, these data suggest that FANCD2 may have

an activity that attenuates transcription-replication collisions during mild replication stress.

### **Role of FANCD2 in suppression of R-loop levels**

Previous studies indicated a role of FANCD2 as well as FANCA in suppression of R-loops in unstimulated HeLa cells [22] or in U2OS cells [23] (without replication stress). Given the apparent role of FANCD2 in transcription-replication coordination at large genes [19], we decided to evaluate R-loop levels in U2OS cells depleted of FANCD2 upon APH treatment using immunohistochemistry with the S9.6 antibody. As in previous studies, and consistent with R-loops being normal components of the human cell genome, we observed high background levels of S9.6 immuno-reactive foci in cells without APH treatment, which was decreased by RNaseH1 expression (Figure 2A). However, a mild increase in the R-loop levels was reproducibly observed in APH stimulated cells, and the levels were further enhanced by siFANCD2 treatment (Figure 2B), implying that FANCD2 may have a protective role in the face of R-loop formation during replication stress. The difference was not huge but we interpret that this could be due to high levels of R-loops as normal components of the genome and replication stress may occur only at some genomic loci such as common fragile sites [19].

### **FANCD2 interacts with RNA processing factor hnRNP U and DDX47**

Mechanistically how FANCD2 affects transcription-replication collisions or suppresses R-loop levels is an important question. It has been suggested that FANCM [22] or FANCI [33] might regulate R-loop levels; however, it remains unclear whether FANCD2 exerts any impact on the activity of these enzymes. We note that some RNA binding proteins have been reported to prevent genome instability and R-loop formation [34]. For example, defects in RNA-processing factors such as ASF/SF2, THO/TREX complex, U2 snRNP, DDX19, DDX23 and other RNA biogenesis factors induce R-loop-associated genome instability [35-40]. Our mass spectrometry analysis indicated that a purified FANCD2 complex isolated from hydroxyurea-treated HeLa S3 cells [7] contained such factors, including hnRNP U and DDX47 (Figure 3A and B). Two recent studies also listed hnRNP U and DDX47 among FANCD2-associated proteins [10,41].

To confirm this observation, we co-immunoprecipitated endogenous hnRNP U or DDX47

with FANCD2 tagged with 3xFLAG from U2OS-D2-FLAG cell lysates. In both cases, we successfully detected the interaction (Figure 4A and B), though it was not appreciably increased after APH treatment. The co-immunoprecipitation (Co-IP) was not mediated by the presence of DNA or RNA since the lysates were treated with Benzonase. Although non-ubiquitinated FANCD2 seemed to be interacting, PLA signals between FANCD2 and hnRNP U or DDX47 were increased following replication stress (Figure 4C and D), suggesting that the mode of interaction could be altered in the replication stress-induced FANCD2 foci.

We excluded the possibility that hnRNP U or DDX47 has an upstream role in FA pathway activation, since siRNA-mediated hnRNP U or DDX47 depletion had no effect on FANCD2 monoubiquitination and foci formation upon mild replication stress (Figure 5A-D). Taken together, we propose that FANCD2 recruits RNA processing factor hnRNP U and DDX47 when FANCD2 accumulates at the sites of transcription-replication conflicts involving R-loops.

### **hnRNP U and DDX47 suppress R-loop levels and function with FANCD2 to resolve transcription-replication collisions**

It has been well established that deregulated or impaired RNA processing results in elevated formation of R-loops [42-44]. Since hnRNP U is an important regulator of splicing, we hypothesized that the hnRNP U bound by FANCD2 at the genomic sites under replication stress has a function to suppress R-loop levels. Indeed, we found that treatment with siRNA against hnRNP U increased the S9.6 foci in both APH-treated and untreated cells (Figure 6A). Similarly, when we depleted DDX47 by siRNA in U2OS cells, increased levels of the S9.6 foci were observed compared to the cells treated with control siRNA (Figure 6B). Given the previously reported role of these factors in RNA metabolism, the elevated levels of R-loops were not surprising. Nonetheless, these observations are consistent with the possibility that FANCD2 may function with hnRNP U and DDX47 in regulation of R-loops.

It has been suggested that the presence of R-loops further exacerbates transcription-replication conflicts by hampering replication fork progression [42-45]. To test whether FANCD2 and hnRNP U or DDX47 function in an epistatic manner, we measured the transcription-replication collision events as PLA signals between PCNA and Pol II. As observed before, *FANCD2*-depleted

cells displayed higher levels of PLA signals after APH treatment. Interestingly, this increase was not further enhanced by the additional depletion of hnRNP U or DDX47, suggesting that FANCD2 and these factors do act in the same pathway to resolve conflicts between transcription and replication upon APH treatment. Taken together, we propose that FANCD2 may recruits RNA processing enzymes, including hnRNP U and DDX47, to resolve transcription-replication collisions during mild replication stress induced by low dose APH treatment. We suggest that the collision might be mitigated by suppression of R-loop formation by the actions of these factors on RNA transcripts.

## Discussion

### FANCD2 functions to suppress R-loop levels upon APH-induced replication stress

It has been shown that cells depleted of FA proteins such as FANCD2, FANCA [19,20], or FANCD1 (BRCA2) [46] displayed increased levels of R-loops in non-stimulated cycling cells. In this study, we showed that R-loop levels were increased in *FANCD2*-depleted cells following APH treatment. We also provided evidence for an interaction of FANCD2 with hnRNP U, an RNA binding protein that functions in transcription elongation [24], pre-mRNA splicing [27], mRNA stability [26] and more broadly, interphase chromatin structures [29]. Given the function of hnRNP U in normal RNA metabolism, it is not surprising that hnRNP U function contributes to the regulation of R-loop levels. BRCA2 interacts with TREX-2, an mRNA export factor [46], and BRCA1 localizes at transcription termination regions with Senataxin [47], an RNA helicase, and these factors might prevent R-loop-mediated genome instability. We suggest that FANCD2 may contribute to pre-mRNA splicing at huge fragile gene loci during replication stress by localizing hnRNP U to the sites of R-loop formation.

We showed that FANCD2 also interacts with DDX47, a member of the DEAD box protein family. The DEAD box proteins are putative ATP-dependent RNA helicases characterized by the conserved Asp-Glu-Ala-Asp (DEAD) motif. Unwinding of the DNA:RNA hybrids by helicases, such as Sen1/Senataxin, DDX19, DDX23, and Pif1, destabilizes R-loops and contributes to maintain genome stability [38,39,47,48].

Our data suggest that DDX47 may belong to this group of helicases. Transcription-



replication collisions induce R-loop accumulation that may further impair progression of replication forks. Based on the above data, we propose that FANCD2 participates in mediation of transcription-replication conflicts via recruitment of these RNA processing factors, and thereby contributes to resolve R-loops, and hence, replication stress. It is interesting to note that a recent study shows an interaction of FANCD2 and FANCI with splicing factor SF3B1, and proposes their role in organizing chromatin domains to ensure coordination of replication and co-transcriptional processes [49].

### **Does FANCD2 also recruit other factors to resolve R-loop formation?**

Other than RNA processing enzymes, the FA pathway might also provide other means for R-loop control. For example, it has been reported that FANCM translocase or FANCI helicase has an activity to resolve DNA: RNA hybrids, and these enzymes may contribute to suppression of R-loop levels. However, it remains unclear how FANCD2 may regulate these FA proteins. On the other hand, BLM helicase, which can also function to resolve DNA:RNA hybrids [50], is known to interact with FANCD2 [51] as well as FANCM.

FANCD2 has been suggested to orchestrate various activities during ICL repair, including protection and restart of the stalled replication forks. FANCD2 promotes HR, perhaps by stabilizing RAD51 filaments or promoting end resection by recruitment of nucleases like CtIP or DNA2. If the HR activity is compromised, the transcription-replication conflicts with stalled forks may persist, leading to genomic sequelae. SMARCA1 and other enzymes promote remodeling and reversal of stalled forks, and then the reversed forks are protected from degradation (mediated by Mre11 or DNA2) by the actions of FA proteins such as FANCD2, BRCA1, BRCA2, and Rad51 [11-14,52]. It seems plausible that these proteins can indirectly suppress R-loop formation/stabilization upon APH-induced replication stress. It would be interesting to test whether the nucleases implicated in the FA pathway, such as FAN1, CtIP, or SLX4 have any role in R-loop regulation in addition to their roles in fork restart or HR activities (end resection or Holliday junction resolution).

In conclusion, our current work has revealed novel FANCD2 interactions with RNA processing factors and their potential involvement in coordination of transcription-replication



conflicts and reduction of R-loop levels (Figure 7). These results may expand the previously suggested roles of FANCD2 as a master regulator/handler of endogenous replication stress.

## Materials and Methods

### Cell culture

U2OS cells were cultured in high glucose DMEM (Nacalai Tesque) supplemented with 10% fetal calf serum (Gibco). Generation of a derivative of U2OS, which incorporated a 3×FLAG tag into the *FANCD2* termination codon has been described previously [19]. For replication stress, cells were treated with 0.4 μM APH for 24 h.

### Antibodies and reagents

The following antibodies were obtained from commercial sources: mouse monoclonal anti-DNA:RNA (S9.6, Kerafast); rabbit polyclonal anti-nucleolin (ab50279, abcam); mouse monoclonal anti-DDDDK-tag (MBL); mouse monoclonal anti-FLAG (M2, Sigma-Aldrich); anti-FLAG M2 magnetic beads (Sigma); normal mouse IgG (Santa Cruz); rabbit polyclonal anti-FANCD2 (Novus); mouse monoclonal anti-PCNA (PC10, Santa Cruz); rabbit polyclonal anti-phospho RNA polymerase II (Ser2, Bethyl); mouse monoclonal anti-hnRNP U (Abcam); rabbit polyclonal anti-DDX47 (Abcam); mouse monoclonal anti-tubulin (T5168, Sigma). Aphidicolin (Wako) and DMSO (Nacalai Tesque) were used at the indicated concentrations. Human GFP-RNaseH1 expression vector was previously described [19].

### siRNA transfections

All siRNA duplexes used were purchased from Invitrogen or Sigma. Transfection and co-transfection were carried out using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. Cells were harvested 72 h after transfection. The individual siRNA duplexes used were:

siFANCD2 (5'-CAGAGUUUGCUUCACUCUCUATT-3') (Invitrogen) [8]; sihnRNP U (5'-GGCCGUGGUAGUUACUCAATT-3') (Invitrogen) [53]; siDDX47 (5'-CUCCAUGCUCUCCUUAACUCCATT-3') (Invitrogen) [54]; siLuciferase Control (siLuc) (5'-

UCGAAGUAUCCGCGUA CGTT-3') (Invitrogen) [55]. Plasmid transfection was done using lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions.

### **Mass spectrometric analysis of purified FANCD2 complexes**

Purification of the FANCD2 complex and analysis by liquid chromatography-tandem mass spectrometry has been described [7].

### **Immunohistochemistry and *in situ* Proximal Ligation Assay (PLA)**

Cells cultured on 15-mm coverslips were washed with PBS and fixed with PBS containing 3% paraformaldehyde, 2% sucrose, 0.5% Triton-X-100, placed on ice for 30 min, and further permeabilized with 0.5% Triton X-100/PBS for 5 min. After blocking with 2% BSA/PBS, cells were stained with the indicated primary antibody for 1 hr at RT. For quantification of the S9.6 foci, cells were pre-extracted with 0.5% Triton X-100/PBS for 5 min, fixed with 4% paraformaldehyde for 10 min, and further extracted with 100% cold methanol at -20°C for 5 min. Slides were blocked with 2% BSA/PBS, then stained with S9.6 antibodies for 1 hr at RT. Alexa Fluor 488-conjugated anti-mouse IgG or Alexa Fluor 594-conjugated anti-rabbit IgG (Molecular Probes) were used as secondary antibodies. PLA was performed with reagents from DuoLink Biosciences, which were used according to the manufacturer's instructions. Images were captured using a BZ-9000 fluorescence microscope (Keyence). Quantification of the PLA signal dots and FANCD2 foci was carried out using Hybrid cell count software (Keyence). The number of S9.6 foci were enumerated using an IN Cell Analyzer 2000 (GE Healthcare). Briefly, the nuclear areas were defined by DAPI staining and then anti-nucleolin positive areas (corresponding to nucleoli) were subtracted. The nuclear S9.6 foci number was determined in the remaining subnuclear areas.

### **Immunoprecipitation and immunoblotting**

Cells were washed once with PBS and lysed in NETN buffer (150 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl pH8.0, 0.5% NP-40) supplemented with protease inhibitor cocktail (Roche), 25 unit/ml Benzonase (Millipore), 2 mM MgCl<sub>2</sub>, and 1 mM Na<sub>3</sub>VO<sub>4</sub>, on ice for 30 min. Then lysates were briefly sonicated and centrifuged at 14,000 rpm for 10 min. FLAG-tagged proteins, were captured using anti-FLAG M2 magnetic beads (Sigma), washed five times with NETN buffer, and analyzed by immunoblotting. Samples were separated by SDS-PAGE, transferred to a PVDF membrane, and detected. The band intensities of the ubiquitinated (L-form) versus non-

ubiquitinated (S-form) FANCD2 were quantified with LAS-4000 ImageQuant TL software (GE Healthcare).

### Statistical analysis

Prism (GraphPad Software) was used to perform unpaired two-tailed Student's *t*-tests.

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### Author contributions.

The most of the experiments were carried out by Y.O. with some help from M.A., M.I. and A. T.-K. except for purification of the FANCD2 complex by A.I. and J.T., and mass spectrometry by M.Taoka and T.I.. M.Takata and Y.O wrote the paper.

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## Figure legends

**Figure 1. Transcription-replication collision and R-loop accumulation are increased by FANCD2-depletion in U2OS cells during mild replication stress**

(A) *In situ* Proximity Ligation Assay (PLA) between PCNA and RNA polymerase II (DMSO, n=66; APH, n=66), PCNA and FANCD2 (DMSO, n=59; APH, n=63), or FANCD2 and RNA



polymerase II (DMSO, n=108; APH, n=101), in U2OS cells treated with 0.4  $\mu$ M APH for 24 hr. In the case of FANCD2/Pol II PLA, U2OS-D2-FLAG cells were used to detect FANCD2 with anti-FLAG antibody. PLA signals per nucleus were plotted. P values were calculated by unpaired, two-tailed Student's *t* test.

(B) PLA signals between PCNA and RNA polymerase II were further increased in *FANCD2*-depleted U2OS cells treated with APH (siLuc DMSO, n=55; siLuc APH, n=56; siFANCD2 DMSO, n=53; siFANCD2, n=53). Means  $\pm$  SEM are shown. P-values were calculated by unpaired, two-tailed Student's *t* test.

### Figure 2. Numbers of S9.6 foci are increased by FANCD2 knock-down

(A) S9.6 foci in U2OS cells upon APH treatment. Cells were transfected with an empty vector or the RNaseH1 expression plasmid then stained with anti-S9.6 and anti-nucleolin after 24 hr (Empty DMSO, n=567; Empty APH, n=514; RNaseH1 DMSO, n=582; RNaseH1 APH, n=561). Nucleolin was stained as a marker for nucleolus which was excluded from counting of the S9.6 foci. Means  $\pm$  SEM are shown. P-values were calculated by unpaired, two-tailed Student's *t* test.

(B) S9.6 foci in U2OS cells depleted of *FANCD2* by siRNA upon APH treatment (siLuc DMSO, n=1535; siLuc APH, n=1494; siFANCD2 DMSO, n=1530; siFANCD2 APH, n=1500). Nucleolin was stained as a marker for nucleolus which was excluded from counting of the S9.6 foci. Means  $\pm$  SEM are shown. P-values were calculated by unpaired, two-tailed Student's *t* test. siFANCD2 or siLuc transfected cells were immunoblotted with anti-FANCD2 or anti-tubulin (below).

### Figure 3. FANCD2 mass spectrometry analysis

A partial list of FANCD2-associated proteins identified in the mass spectrometry analysis previously carried out by Unno et al. [7]. Cells were treated with or without 2 mM HU (hydroxyurea) for 24 hr and subjected to purification of FANCD2 complex.

### Figure 4. FANCD2 interaction with RNA processing factor hnRNP U and DDX47

(A-B) Co-IP of FANCD2-FLAG and endogenous hnRNP U and DDX47 from U2OS-D2-FLAG cells.

(C-D) *In situ* PLA between FANCD2 and hnRNP U in U2OS cells (DMSO, n=51; APH, n=50) or between FANCD2-FLAG and DDX47 in U2OS-D2-FLAG cells treated with APH (DMSO, n=50; APH, n=51). Means  $\pm$  SEM are shown. P-values were calculated by unpaired, two-tailed Student's *t* test.

### Figure 5. hnRNP U and DDX47 depletion do not affect FA pathway activation

(A-D) hnRNP U or DDX47 depletion by siRNA had no effect on FANCD2 monoubiquitination and foci formation in U2OS cells treated with 0.4  $\mu$ M APH for 24 hr (siLuc DMSO, n=67; siLuc APH, n=68; siHNRNP U DMSO, n=63; siHNRNP U APH, n=74 in (A)) (siLuc DMSO, n=53; siLuc APH, n=54; siDDX47 DMSO, n=56; siDDX47 APH, n=49 in (C)). Mean number of foci per nucleus  $\pm$  SEM are shown. P-values were calculated by unpaired, two-tailed Student's *t* test.

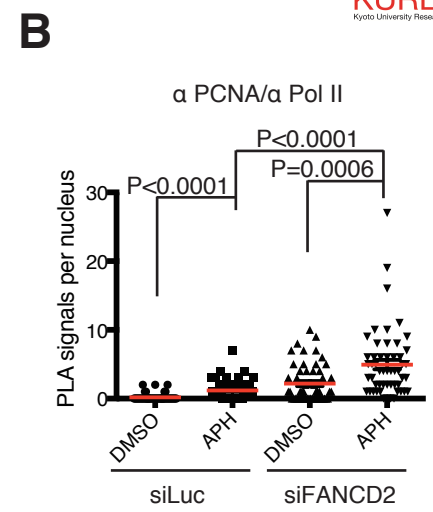
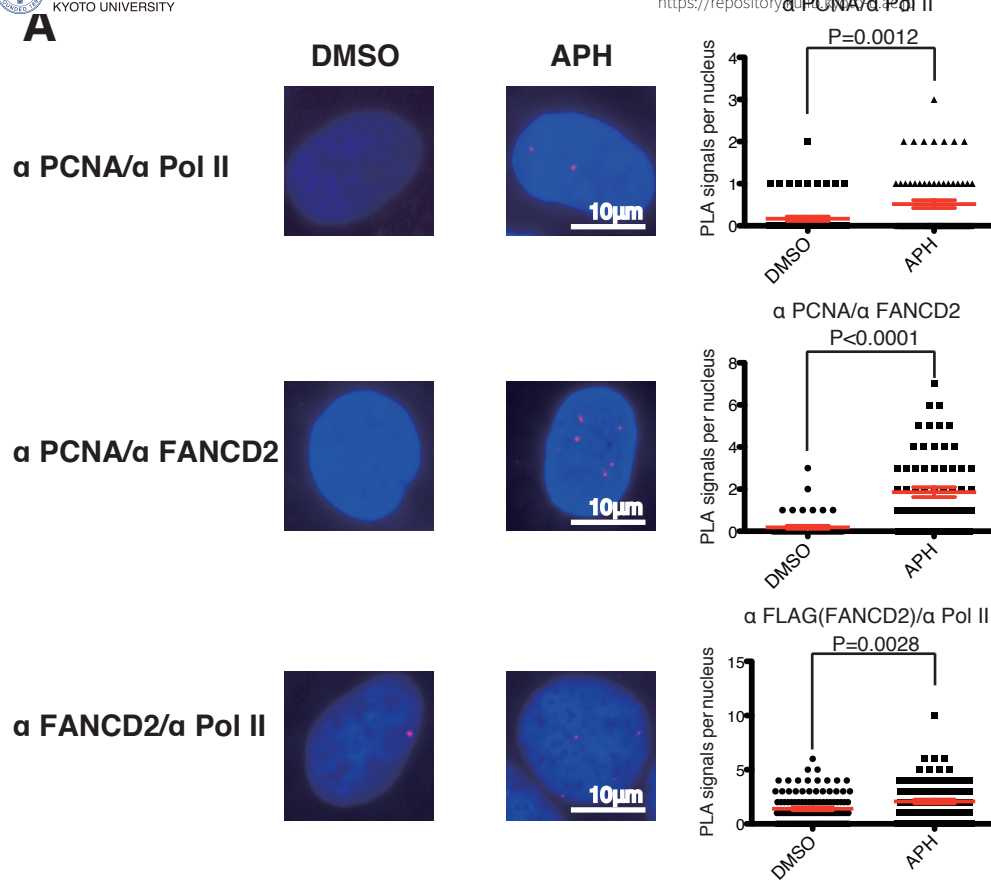
### Figure 6. Numbers of S9.6 foci were increased by hnRNP U and DDX47 siRNA knock down

(A-B) *hnRNP U* or *DDX47*-depleted U2OS cells displayed increased numbers of S9.6 foci (siLuc DMSO, n=1615; siLuc APH, n=1663; siHNRNP U DMSO, n=2320; siHNRNP U APH, n=1745 in (A)) (siLuc DMSO, n=1444; siLuc APH, n=1308; siDDX47 DMSO, n=1393; siDDX47 APH, n=1384 in (B)). The counting of S9.6 foci was carried out as in Figure 2. Means  $\pm$  SEM are shown. P-values were calculated by unpaired, two-tailed Student's *t* test.

(C) PLA signals between PCNA and RNA polymerase II were not increased significantly in *FANCD2* and *HNRNP U* or *DDX47* doubly-depleted U2OS cells compared to *FANCD2*-depleted cells (siLuc DMSO, n=104; siLuc APH, n=107; siHNRNP U APH, n=108; siHNRNP U+siFANCD2 APH, n=109; siDDX47 APH, n=111; siDDX47+siFANCD2 APH, n=107; siFANCD2 APH, n=109), suggesting that all three proteins function in the same pathway for reducing transcription-replication conflicts. Means  $\pm$  SEM are shown. P-values were calculated by two-tailed Student's *t* test.

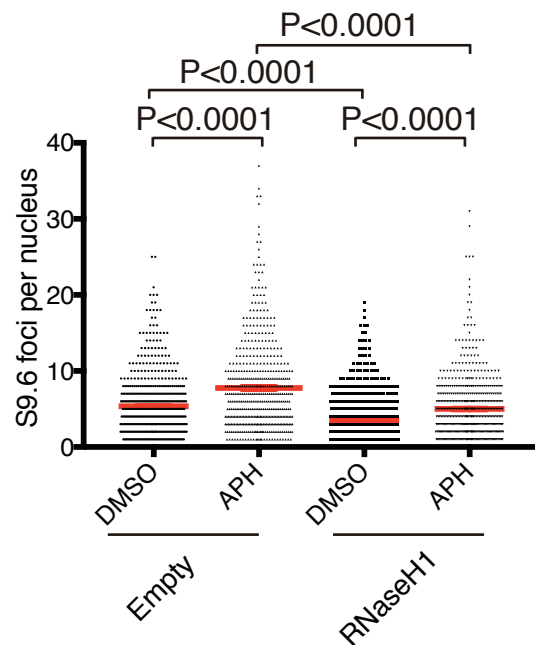
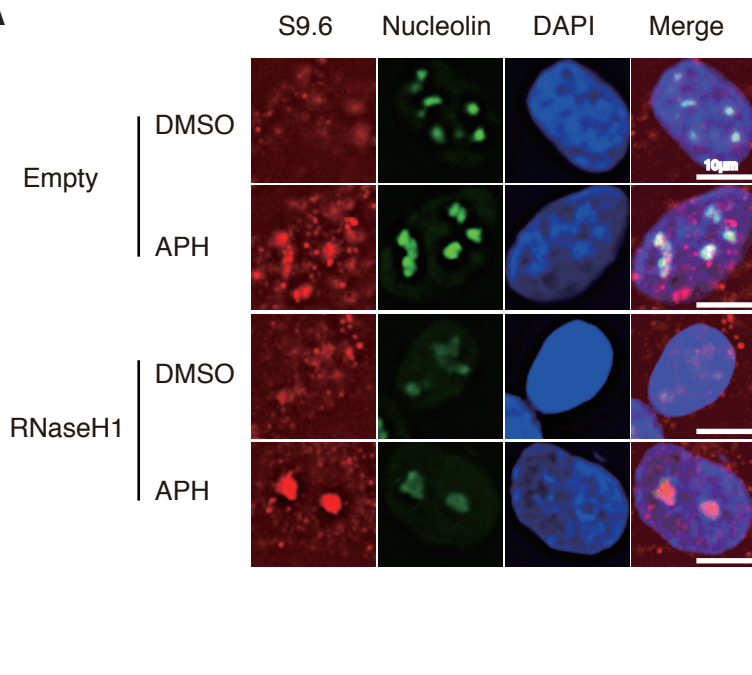
### Figure7. A scheme depicting FANCD2 function in R-loop resolution

- (i) FANCD2 accumulates at large transcribed genes, including CFSs. Its accumulation depends on R-loops induced by transcription-replication collisions during mild replication stress.
- (ii) FANCD2 recruits RNA processing enzymes including hnRNP U and DDX47 to resolve R-loops.
- (iii) FANCD2 may also contribute to stalled fork restart by resolving R-loops.



**Figure 1**

A



B

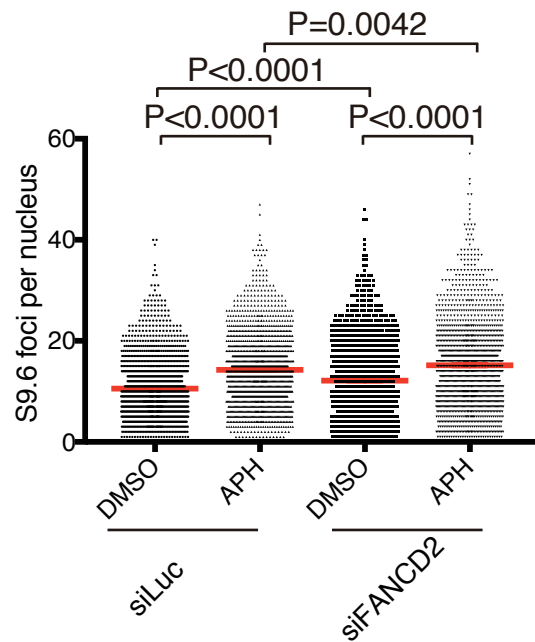
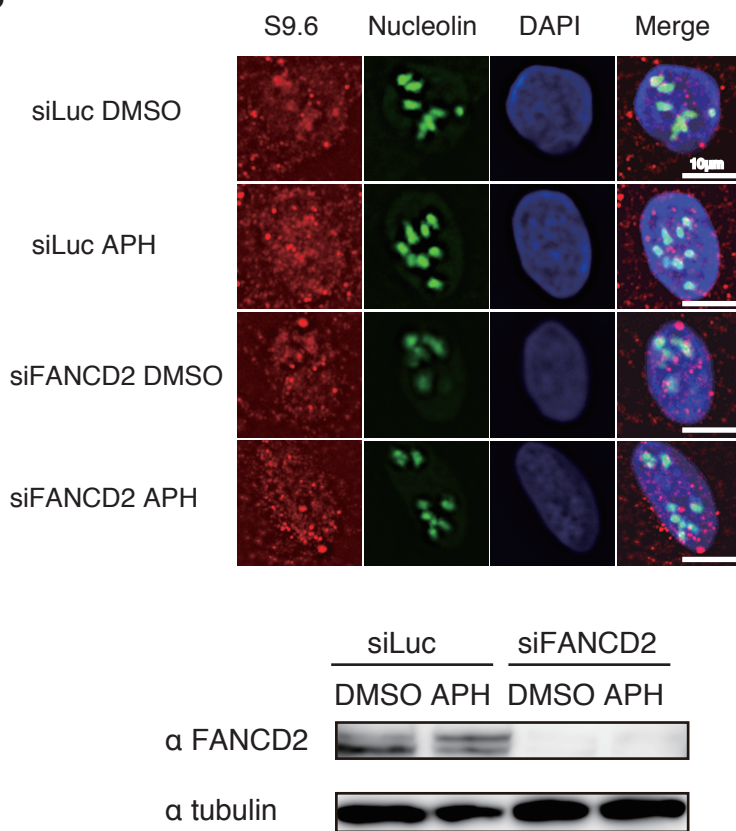


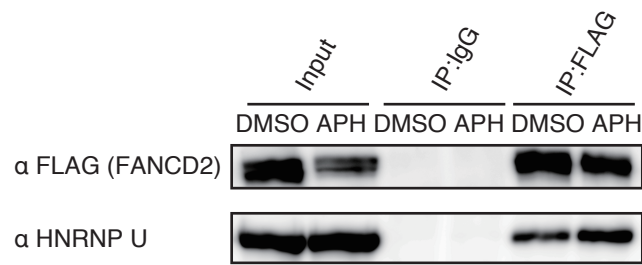
Figure 2

## FANCD2-associated proteins

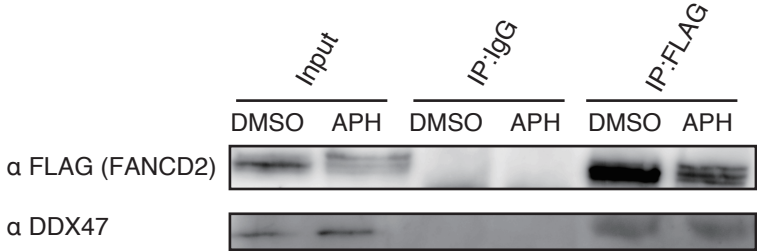
Genes	Peptide # HU(-)	Peptide # HU(+)
DDX47	3	5
hnRNP U	-	2
Matrin3	-	1
SF3B3	-	1
hnRNP R	-	1

Figure 3

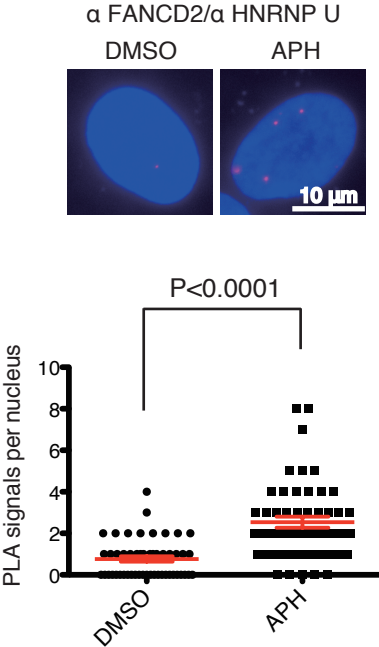
A



B



C



D

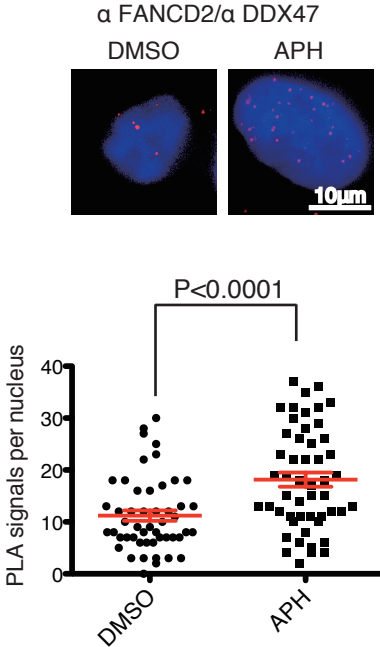
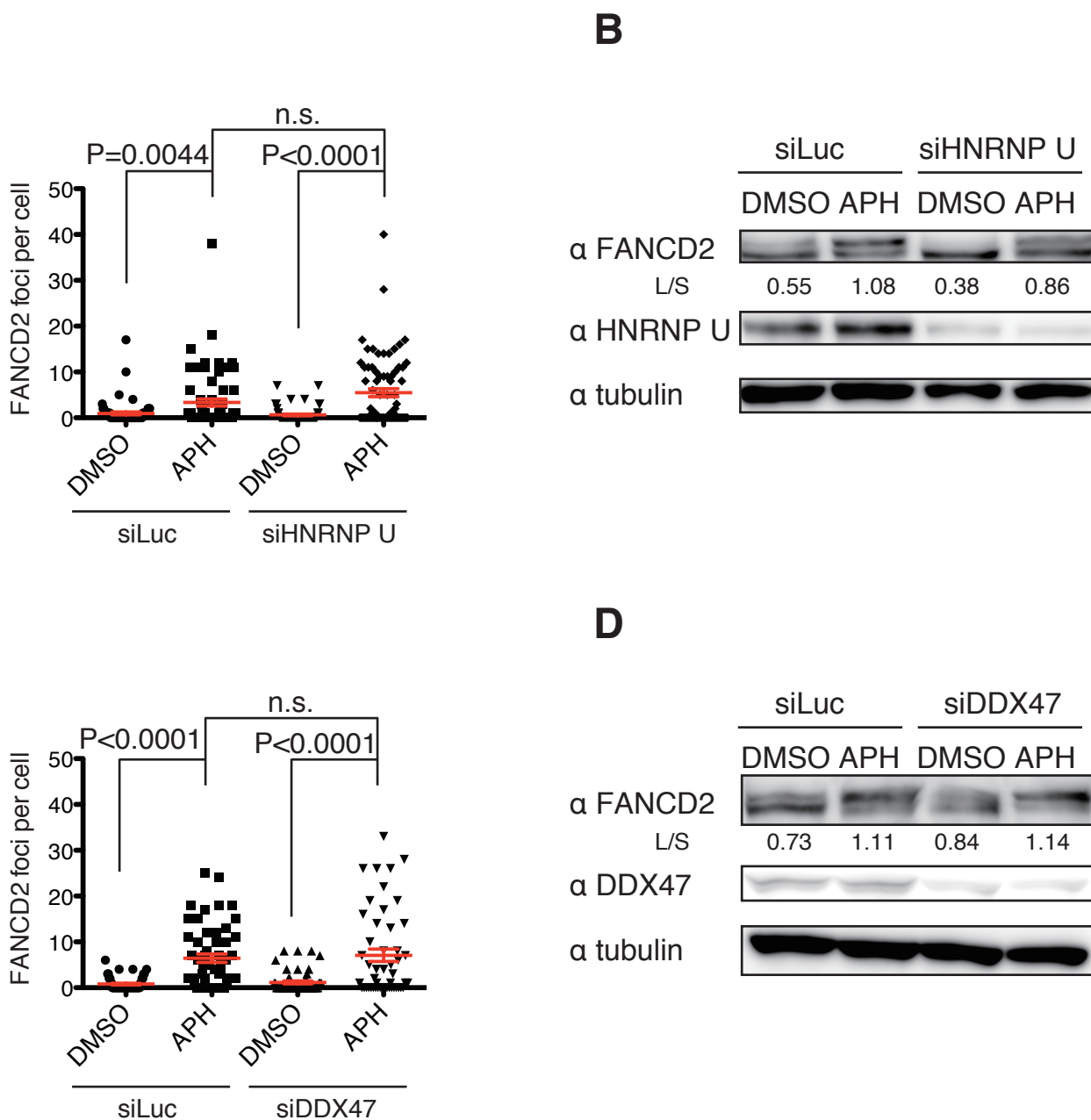


Figure 4



**Figure 5**



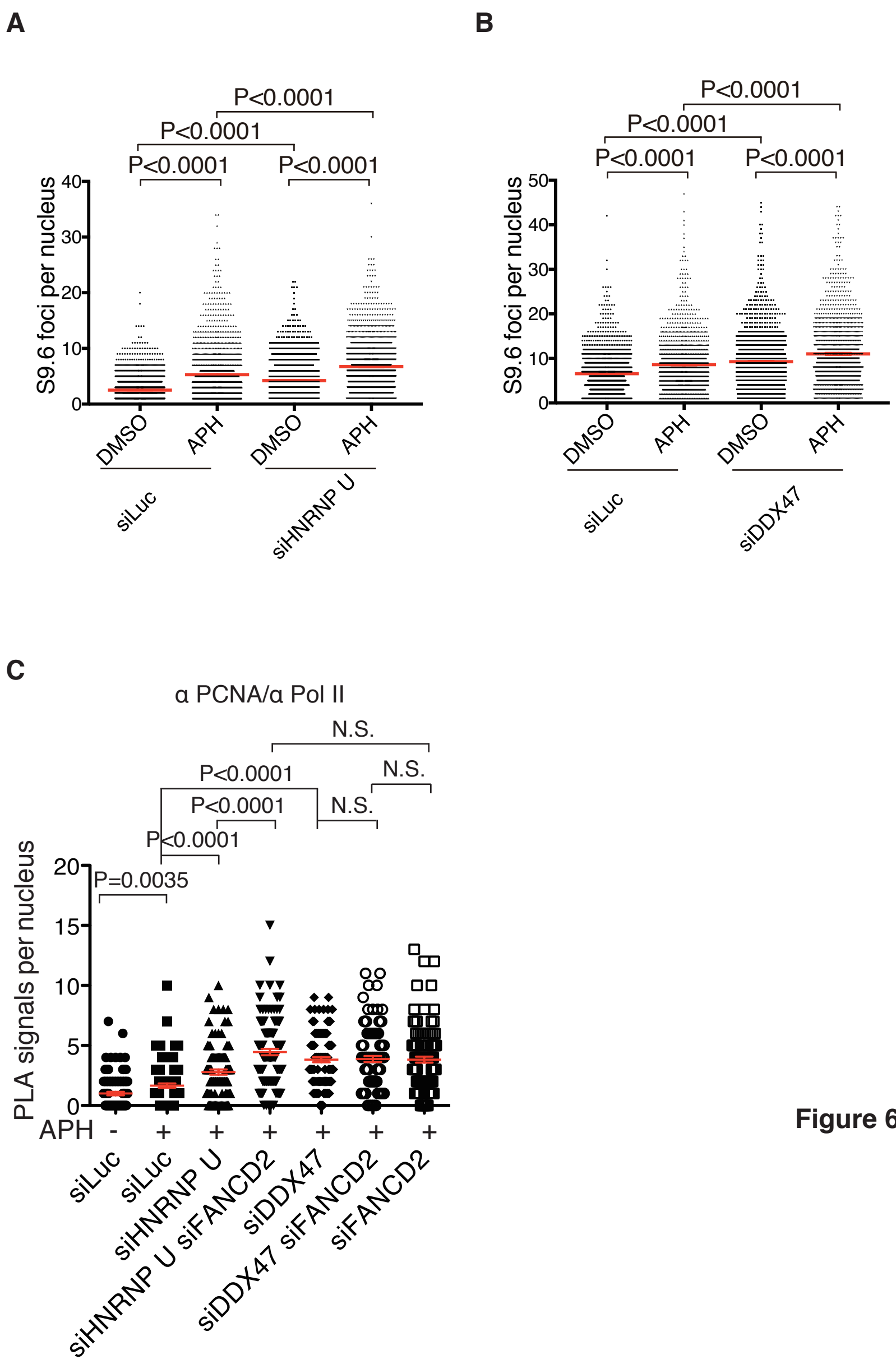
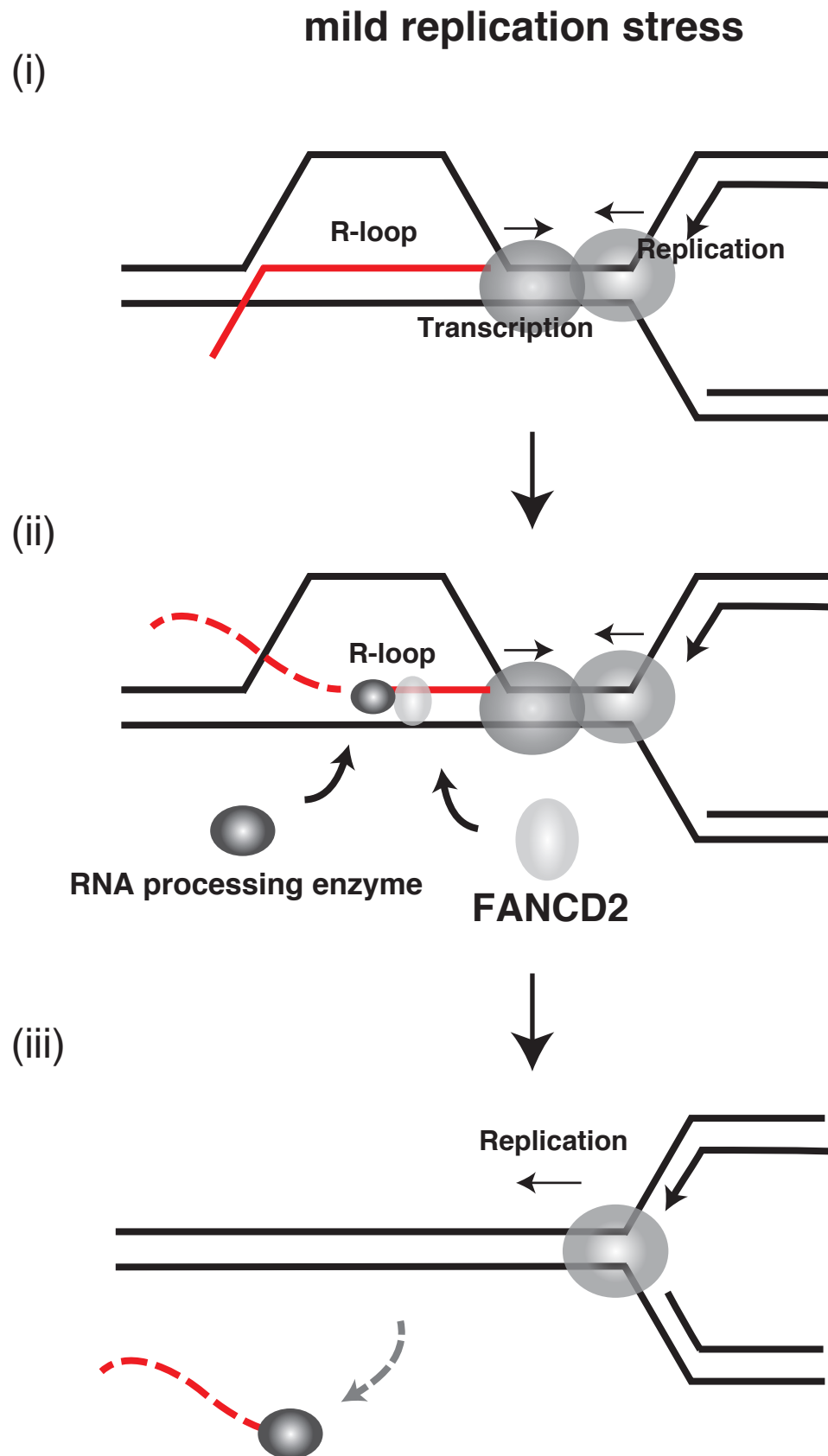


Figure 6



**Figure 7**